GENES AND PROTEINS, AND THEIR USE

Field of the Invention

This invention relates to the identification of bacterial genes and proteins, and their use. More particularly, it relates to their use in therapy, for immunisation and in screening for drugs.

Background to the Invention

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, is the causative agent of various conditions. In particular, GBS causes:

Early onset neonatal infection.

This infection usually begins *in utero* and causes severe septicaemia and pneumonia in infants, which is lethal if untreated and even with treatment is associated with a 10-20% mortality rate.

Late onset neonatal infection.

This infection occurs in the period shortly after birth until about 3 months of age. It causes a septicaemia, which is complicated by meningitis in 90% of cases. Other focal infections also occur including osteomyelitis, septic arthritis, abscesses and endopthalmitis.

Adult infections.

These appear to be increasingly common and occur most frequently in women who have just delivered a baby, the elderly and the immunocompromised. They are characterised by septicaemia and focal infections including osteomyelitis, septic arthritis, abscesses and endopthalmitis.

Urinary tract infections.

GBS is a cause of urinary tract infections and in pregnancy accounts for about 10% of all infections.

Veterinary infections.

GBS causes chronic mastitis in cows. This, in turn, leads to reduced milk production and is therefore of considerable economic importance.

GBS infections can be treated with antibiotics. However, immunisation is preferable. It is therefore desirable to develop an immunogen that could be used in a therapeutically-effective vaccine.

Summary of the Invention

The present invention is based on the identification of a series of genes in GBS, and also related organisms, the products of which may be localised on the outer surface of the organism and therefore may be used as a target for immuno-therapy.

According to one aspect of the invention, a peptide is encoded by an operon including any of the genes identified herein as pho1-13, pho3-21, pho2-15, pho3-18, pho3-22, pho3-3, pho3-17, pho2-2, pho1-5, pho3-1, pho3-23, pho3-50, pho1-14, pho2-10, pho3-14, pho3-24 and pho3-29, obtainable from Group B *Streptococcus*, or a homologue or functional fragment thereof. Such a peptide is suitable for therapeutic use, e.g. when isolated.

The term "functional fragments" is used herein to define a part of the gene or peptide which retains the activity of the whole gene or peptide. For example, a functional fragment of the peptide may be used as an antigenic determinant, useful in a vaccine or in the production of antibodies.

A gene fragment may be used to encode the active peptide. Alternatively, the gene fragment may have utility in gene therapy, targetting the wild-type gene *in vivo* to exert a therapeutic effect.

A peptide according to the present invention may comprise any of the amino acid sequences identified herein as SEQ ID NOS. 2, 4, 6, 8, 10, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33 and 35, or a functional fragment thereof.

Because of the extracellular or cell surface location, the peptides of the present invention may be suitable candidates for the production of therapeutically-effective vaccines against GBS. The term "therapeutically-effective"

is intended to include the prophylactic effect of vaccines. For example, a vaccine may comprise a peptide according to the invention, or the means for its expression, for the treatment of infection. The vaccine may be administered to females prior to or during pregnancy to protect mother and neonate against infection by GBS.

According to another aspect of the invention, the peptides or genes may be used for screening potential antimicrobial drugs or for the detection of virulence.

A further aspect of this invention is the use of any of the products identified herein, for the treatment or prevention of a condition associated with infection by a Group B Streptococcal strain.

Although the protein has been described for use in the treatment of patients, veterinary uses of the products of the invention are also considered to be within the scope of the present invention. In particular, the peptides or the vaccines may be used in the treatment of chronic mastitis, especially in cows.

Description of the Invention

The present invention is described with reference to Group B Streptococcal strain M732. However, all the GBS strains and many other bacterial strains are likely to include related peptides or proteins having amino acid sequence homology with the peptide of M732. Organisms likely to contain the peptides include, but are not limited to, *S. pneumoniae*, *S. pyogenes*, *S. suis*, *S. milleri*, Group C and Group G *Streptococci* and *Enterococci*. Vaccines to each of these may be developed in the same way as described for GBS.

Preferably, the peptides that may be useful for the production of vaccines have greater than 40% sequence similarity with the peptides identified herein. More preferably, the peptides have greater than 60% sequence similarity. Most preferably, the peptides have greater than 80% sequence similarity, e.g. 95% similarity.

Having characterised a gene according to the invention, it is possible to use the gene sequence to establish homologies in other microorganisms. In this way it is possible to determine whether other microorganisms have similar outer surface products. Sequence homologies may be established by searching in existing databases, e.g. EMBL or Genbank.

Peptides or proteins according to the invention may be purified and isolated by methods known in the art. In particular, having identified the gene sequence, it will be possible to use recombinant techniques to express the genes in a suitable host. Active fragments and homologues can be identified and may be useful in therapy. For example, the peptides or their active fragments may be used as antigenic determinants in a vaccine, to elicit an immune response. They may also be used in the preparation of antibodies, for passive immunisation, or diagnostic applications. Suitable antibodies include monoclonal antibodies, or fragments thereof, including single chain fv fragments. Methods for the preparation of antibodies will be apparent to those skilled in the art.

The preparation of vaccines based on attenuated microorganisms is known to those skilled in the art. Vaccine compositions can be formulated with suitable carriers or adjuvants, e.g. alum, as necessary or desired, and used in therapy, to provide effective immunisation against Group B Streptococci or other related microorganisms. The preparation of vaccine formulations will be apparent to the skilled person.

More generally, and as is well known to those skilled in the art, a suitable amount of an active component of the invention can be selected, for therapeutic use, as can suitable carriers or excipients, and routes of administration. These factors will be chosen or determined according to known criteria such as the nature/severity of the condition to be treated, the type or health of the subject etc.

The products of the present invention were identified as follows:

A partial gene library of GBS (strain M732) chromosomal DNA was prepared using the plasmid vectors pFW-*phoA*1, pFW-*phoA*2 and pFW-*phoA*3 (Podbielski, A. *et al*. 1996. Gene 177:137-147). These plasmids possess a constitutive spectinomycin adenyltransferase antibiotic resistance marker, which confers a high level of spectinomycin resistance and is therefore easily selected. Furthermore, these vectors contain a truncated (leaderless) *Escherichia coli phoA* gene for alkaline phosphatase. The three vectors differ only with respect to the reading frame in which the leaderless *phoA* gene exists, as compared to an upstream in-frame *Bam*HI restriction enzyme site. Because this truncated *E. coli phoA* gene lacks the appropriate leader sequence for export of this enzyme across the bacterial membrane, extracellular alkaline phosphatase activity is absent when these plasmids are propagated in an *E. coli phoA* mutant (e.g. strain DH5α). The chromogenic alkaline phosphatase substrate, XP (5-bromo-4-chloro-3-indolyl-phosphate), does not enter intact bacterial cells and therefore only exported or surface associated alkaline phosphatase activity can be detected. When exported or surface associated alkaline phosphatase activity is present, the chromogenic XP substrate is cleaved to yield a blue pigment and the corresponding bacterial colonies can be identified by their blue colour.

Plasmid DNA was digested to completion with *Bam*HI and dephosphorylated using shrimp alkaline phosphatase. GBS genomic DNA was partially digested with *Sau*3AI, size fractionated on a sucrose gradient and fragments <1kb in size were ligated into the prepared pFW-*phoA* vectors. *E. coli* strain DH5α was chosen as the cloning host since it lacks a functional *phoA* gene. Recombinant plasmids were selected on Luria agar containing 100 μg/ml of spectinomycin and 40 μg/ml of the chromogenic XP substrate. *E. coli* transformants harbouring plasmids containing GBS

insert DNA that complements the export signal sequence of the leaderless *phoA* gene were identified by the blue colour of the colonies. Approximately 30000 different recombinant plasmids containing GBS insert DNA were screened in this manner and 83 recombinant plasmids, which complemented the leaderless *phoA*, were chosen for further study.

From these experiments, several clones were selected each containing a plasmid containing a gene (or part thereof), which complemented the leaderless *phoA*.

Having identified the gene in each clone it is then possible to obtain the full-length gene sequence, as follows.

Using the identified and sequenced gene fragment, oligonucleotide primers were designed for genomic DNA sequencing. These primers were designed so as to sequence in an 'outward' direction from the obtained sequence. Once read, the sequence obtained was checked to see if the 5' and 3' termini of the gene had been reached. The presence of these features was identified by checking against homologous sequences, and for the 5' end the presence of an AUG start codon (or accepted equivalent) preceded by a Shine-Dalgarno consensus sequence, and for the 3' end, the presence of a translation termination (Stop) codon.

Upon identification of the full-length gene, primers were designed for amplification of full-length product. Primers used included restriction enzyme recognition sites (NcoI at the 5'end and Eco0109I at the 3' end) to allow subsequent cloning of the product into the Lactococcal expression system used.

PCR was carried out using the primers, and the products cloned into a pCR 2.1 cloning vector (In Vitrogen). Following confirmation of the presence of the cloned fragment, the DNA was excised using the restriction enzymes NcoI and Eco0109I.

The vector into which this fragment was inserted was a modified version of pNZ8048 (Kuipers, O. P. *et al*. (1998) J. Biotech 64: 15-21). This vector, harbouring a

lactococcal origin of replication, a chloramphenicol resistance marker, an inducible nisin promoter and a multicloning site was altered by the replacement of the multicloning site with two 10X His tags, flanked on the 5-most end with an NcoI site, split in the middle with a multicloning site (including an Eco0109I site), and a Stop (termination) codon at the 3'end of the His tags.

The gene of interest was inserted so that a 10X His tag was in the 3' position relative to the coding region. Following transformation of the recombinant plasmid into L.lactis (strain NZ9000 - Kuipers, O. P. *et al.* (1998) *supra)*, a 400 ml liquid culture was set up and translation of the protein was induced by the addition of nisin to the culture. After a 2 hour incubation, the cells were harvested and lysed by bead beating. The resultant lysate was cleared by centrifugation, then passed over a metal affinity (Talon, Clonetech) column. The column was washed repeatedly before bound proteins were eluted with Imidazole.

To identify fractions containing the His-tagged recombinant protein, an aliquot from each fraction was analysed by SDS-PAGE, Western blotted and probed with anti-His antibodies.

The recombinant protein obtained was then used to immunise New Zealand white rabbits, with pre-immune sera being harvested prior to immunisation. Following a boost, the rabbits were sacrificed and sera collected. This sera was used in Western blots, ELISA and animal protection models.

Using the sera obtained from the animal studies, immunosorption studies were carried out.

Group B *Streptococcus* was grown in 20ml Todd Hewitt broth (THB) for 8 hours, harvested and resuspended in 5ml PBS. 50μl aliquots of this were used to coat wells in a 96 well plate (Nunc Immuno-Sorb). This was left at 4°C overnight to allow for adsorbance of the bacteria onto the plate. Plates were washed twice with PBS, then blocked

with 3%BSA in PBS for 1hr at 37°C. Plates were again washed. Serial 10 fold dilutions of the sera were made in PBS and 50μl of these dilutions were added to the wells of the plate, in duplicate. The plate was covered and incubated for 1 hr at 37°C. The plate was washed, then 50μl anti-rabbit alkaline phosphatase conjugated secondary antibody at a concentration of 1:5000 was added to each well. Following incubation at 37°C for an hour, the plate was washed again. 50μl substrate (PNPP) was added to each well, and the reaction allowed to proceed for 30min before the adsorbance was read at 405 nm.

Animal protection studies were also carried out to test the effectiveness of protection on the immunised rabbits.

GBS M732 was grown up in THB until mid-log phase was reached - approximately 5 hours. Cells were counted in a counting chamber, and bacteria were diluted to give a concentration of $2x10^7$ bacteria per ml in pre-immune or test sera. 50μl of this was injected via the intraperitoneal route into 0-1 day old mice. The mice were observed for survival over 48 hours.

The following Examples illustrate the invention.

Example 1

A first clone contained a gene sequence identified herein as SEQ ID NO. 1, with an amino acid sequence identified as SEQ ID NO. 2, and classified as pho1-13.

A comparison of the amino acid sequence of pho1-13 was performed.

Homologues to the GBS pho1-13 gene product can be identified in *Streptococcus pyogenes*, *S. pneumoniae*, *S. salivarius*, *Escherichia coli*, *Yersinia enterocolitica*, *Aquifex aeolicus*, *Helicobacter pylori* and *Haemophilus influenzae*. The *S. pyogenes* and *S. pneumoniae* homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. In all other cases, the above homologues

can be identified as ATP-dependent Clp protease proteolytic subunits. The catalytic activity of Clp proteases results in the hydrolysis of proteins to small peptides in the presence of ATP and magnesium (Giffard, P.M. *et al*. 1993. J. Gen. Microbiol. 139:913-920). Furthermore, the ClpP component of Clp proteases has been shown to be induced as part of the heat shock response (Kroh, H.E. and L.D. Simon. 1990. J. Bacteriol. 172:6026-6034) and it is probable that this subunit or the complete proteolytic domain would associated with the bacterial surface.

Immunisation studies, carried out as described above, yielded the following results.

Treatment	No animals	No animals surviving at time (hrs)	
		24	48
PBS	10	7	0
Pre-immunised	37	13	0
Immunised	38	17	9

Example 2

A second clone was selected containing a plasmid designated pho1-14. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences are shown as SEQ ID NOS. 3 and 4, respectively.

A comparison of the amino acid sequence of pho1-14 was performed.

Homologues to the GBS pho1-14 gene product can be identified in *Streptococcus pyogenes*, *Enterococcus faecalis* and *Streptococcus pneumoniae*. These homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. Additionally, two possible homologues were also

identified from *Shigella flexneri* (SpaR) and *Yersinia pseudotuberculosis* (YscT). These latter two homologues are related proteins, believed to be anchored in the bacterial membrane (Bergman, T. *et al.* 1994. J. Bacteriol. **176:2619-2626**). In *S. flexneri*, the product of the *spaR* gene has been shown to be important for invasion of epithelial cells (Sasakawa, C. *et al.* 1993. J. Bacteriol. **175:2334-2346**). Furthermore, the product of the *spaR* gene is also required for surface presentation of invasion plasmid antigens. The analogous protein in *Y. pseudotuberculosis* is a component of the Yop secretion system and is also important for virulence in this organism.

Example 3

A third clone was selected containing a plasmid designated pho1-5. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences are shown as SEQ ID NOS. 5 and 6.

A comparison of the amino acid sequence of pho1-5 was performed.

Homologues to the GBS pho1-5 gene product can only be identified in *Streptococcus pyogenes* and *Staphylococcus carnosus* (sceA). The *S. pyogenes* homologue was identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. Furthermore, little information is available on the function of the *sceA* gene product from *S. carnosus*. The *sceA* gene product shows some sequence similarity to the aggregation promoting protein from *Lactobacillus gasseri*. Based on analysis of the *sceA* gene product, this molecule contains a well-conserved signal sequence and is apparently secreted or associated with the bacterial cell surface.

Example 4

A further clone was selected containing a plasmid designated pho3-3. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The

nucleotide and deduced amino acid sequences are shown as SEQ ID NOS. 7 and 8.

A comparison of the amino acid sequence of pho3-3 was performed.

Homologues to the GBS pho3-3 gene product can be identified in *Streptococcus mutans* (rmiC), (cpsM) *S. pneumoniae* and *S. pyogenes*. The *S. pyogenes* homologue was identified from genome sequence data and no annotations were available as to the identity of the gene or gene product. In *S. pneumoniae*, the homologue can be identified as dTDP-4-keto-6-deoxy glucose-3,5-epimerase. In the other two cases, the above homologues can be identified as dTDP-4-keto-L-rhamnose reductase (rmlC). In *S. mutants*, the gene encoding this enzyme, *rmlC*, is part of the *rml* locus. The *rml* locus consists of three genes which exhibit significant similarity to enzymes involved in the biosynthesis of dTDP-rhamnose, the immediate precursor of the rhamnose component in the *S. mutans* polysaccharide capsule (Tsukioka, Y. *et al*. 1997. J. Bacteriol. **179**:1126-1134). An analogous locus has also been identified in *S. pneumoniae* (Coffey, T.J. *et al*. 1998. Mol. Micobiol. **17**:73-83). Almost all *Streptococci* characteristically possess rhamnose in their cell wall associated polysaccharides (Schleifer, K.H. and R. Kilper-Bälz. 1987. Syst. Appl. Microbiol. **10**:1-19), and it is highly probable that dTDP-4-keto-L-rhamnose reductase would be associated with the outer surface in *Streptococci*.

Example 5

A further clone was selected containing a plasmid designated pho2-10. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*.

The nucleotide sequence is shown as SEQ ID NO. 9. From this, upstream and downstream coding regions were identified, and the deduced amino acid sequences shown as SEQ ID NOS. 10 and 11.

A comparison of the amino acid sequences of pho2-10 was performed.

Homologues to the GBS pho2-10 gene product can be identified in *Streptococcus pyogenes*, *Enterococcus faecalis*, *Debaryomyces occidentalis* (hatI) and *Escherichia coli* (trkD). The *S. pyogenes* and *E. faecalis* homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. In the yeast *D. occidentalis*, the *hak1* gene is a homologue of the *trkD* gene from *E. coli* (Banuelos, M.A. *et al.* 1995. EMBO J. 14:3021-3027). The *trkD* gene of *E. coli* is part of the *kup* potassium uptake system. The specific homolog identified here is the *kup* system potassium uptake protein. The *kup* system is a constitutive potassium uptake system in *E. coli*. The *kup* system potassium uptake protein contains a highly hydrophobic N-terminus that is predicted to span the membrane at least 12 times. Kup is not homologous to other known membrane protein sequences. There is no indication of ATP binding, and it is proposed that the system is driven by a chemiosmotic gradient (Schleyer, M. & E.P. Bakker, 1993. J. Bacteriol. 175:6925-6931).

Example 6

A further clone was selected containing a plasmid designated pho2-15. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 12 and 13.

A comparison of the amino acid sequence of pho2-15 was performed.

Homologues to the GBS pho2-15 gene product can be identified in *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Escherichia coli* (gatC and SgcC). The *S. pyogenes*, *S. pneumoniae* and *E. faecalis* homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. In *E. coli*, the *gatC* and *sgcC* gene products can be identified as being the IIC component of phosphoenolypyruvate-dependent sugar

phosphotransferase systems (PTS), a major carbohydrate active-transport system. In PTS systems, the IIC component is typically involved in binding of extracellular carbohydrates and forms a complex with the IID component to constitute a membrane channel (Nobelmann, B. and J.W. Lengeler. 1995. Biochim. Biophys. Acta 1262:69-72).

Example 7

A further clone was selected containing a plasmid designated pho2-2. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 14 and 15, respectively.

A comparison of the amino acid sequence of pho2-2 was performed.

Homologues to the GBS pho2-2 gene product can be identified in *Enterococcus faecalis, Escherichia coli* (malK and afuC), *Bacillus subtilis* (glnO), *Haemophilus influenzae* (yebM and potA), *Streptococcus pyogenes, Streptococcus pneumoniae* and *Salmonella typhimurium* (malK). The *E. faecalis, S. pyogenes* and *S. pneumoniae* homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. In all other cases, homologues represented ATP-binding transport proteins that are part of ABC type transporters. Many of the components of ABC type transporters are membrane or cell surface associated, as these systems are involved in the transport of macromolecules from the extracellular environment to the intracellular compartment.

Example 8

A further clone was selected containing a plasmid designated pho3-14. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 16 and 17.

A comparison of the amino acid sequence of pho3-14 was performed and no homologues could be identified in any of

the public databases. One homologue to the GBS pho3-14 gene product can be identified in *Streptococcus pyogenes*, but this homologue was identified from genome sequence data and no annotations were available as to the identity of the gene or gene product. Using this *S. pyogenes* homologue to search the public databases yielded no further information. Since the pho3-14 product complemented the leaderless *phoA* gene, it can be concluded that this protein (or part thereof) would most probably be located extracellularly.

Example 9

A further clone was selected containing a plasmid designated pho3-17. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 18 and 19.

A comparison of the amino acid sequence of pho3-17 was performed.

Homologues to the GBS Pho3-17 gene product can be identified in *Streptococcus mutans* and *Lactococcus lactis*, with similarity being shown to N-acetyl muramidase. Similarity is also seen with an unidentified gene, yubE from *Bacillus subtilis*.

N-acetylmuramidase is an autolysin that is involved in cell division. Using this limited information along with the fact that pho3-17 complemented the leaderless *phoA* gene, it can be concluded that the pho3-17 product would most probably be located extracellularly.

Example 10

A further clone was selected containing a plasmid designated pho3-18. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 20 and 21.

A comparison of the amino acid sequence of pho3-18 was performed.

Homologues to the GBS pho3-18 gene product can be identified in *Streptococcus pyogenes* and *Streptococcus*

pneumoniae. These homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. Using these *S. pyogenes* and *S. pneumoniae* homologues to search the public databases showed some similarity to outer surface and membrane spanning proteins. Since the ORF3-18 product complemented the leaderless *phoA* gene, it can be concluded that this protein (or part thereof) would most probably be located extracellularly.

Example 11

A further clone was selected containing a plasmid designated pho3-1. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 22 and 23.

A comparison of the amino acid sequence of pho3-1 was performed.

Homologues to the GBS pho3-1 gene product can be identified in *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Bacillus subtilis* (yutD) and *Enterococcus faecalis*. The *S. pyogenes*, *S. pneumoniae* and *E. faecalis* homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. In *B. subtilis*, the function of the *yutD* gene product is unknown. It can be noted however, that the *yutD* gene is located on the *B. subtilis* chromosome in a region containing genes involved in cell wall synthesis. The fact that this DNA sequence complemented the leaderless *phoA* gene suggests that this gene product is extracellularly located.

Example 12

A further clone was selected containing a plasmid designated pho3-21. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 24 and 25.

A comparison of the amino acid sequence of pho3-21 was performed.

Homologues to the GBS pho3-21 gene product can be identified in *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Lactobacillus fermentum* (bspA) and *Lactobacillus reuteri* (cnb). The *S. pyogenes* and *S. pneumoniae* homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. In *L. fermentum*, the *bspA* gene product has been identified as being a basic cell surface-located protein that has some sequence similarity to family III of the bacterial solute-binding proteins (Turner, M.S. *et al.* 1997. J. Bacteriol. **179**:3310-3316). In *L. reuteri*, the *cnb* gene product has been identified as a collagen binding protein that has some sequence similarity to the solute-binding component of bacterial ABC transporters (Roos, S. *et al.* 1996. FEMS Microbiol. Lett. **144**:33-38).

Example 13

A further clone was selected containing a plasmid designated pho3-22. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 26 and 27.

A comparison of the amino acid sequence of pho3-22 was performed.

Homologues to the GBS pho3-22 gene product can be identified in *Enterococcus faecalis*, *Streptococcus equisimilis* (lppC), *Pseudomonas fluorescens* (oprI) and *Streptococcus thermophilus* (orf142). The *E. faecalis* homolog was identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. In *S. equisimilis*, the *lppC* gene product has been identified as being a lipoprotein that is homologous to the E(P4) outer membrane protein from *Haemophilus influenzae* (Gase, K. *et al.* 1997. Med. Microbiol. Immunol. **186**:63-73). Likewise, the *P.*

fluorescens oprI gene encodes a major outer membrane lipoprotein (Cornelis, P. *et al*. 1989. Mol. Microbiol. 3:421-428). In *S. thermophilus*, the orf142 product has been putatively identified as a cell surface exposed lipoprotein that may act as a receptor for the bacteriophages TP-J34 and Sfi21 (Neve, H. *et al*. 1998. Virology 241:61-72). The ORF3-22 product showed good similarity to the above homologues, particularly at the N-terminus. This is most likely the region required for complementation of the leaderless *phoA* gene, and therefore serves as a leader sequence.

Example 14

A further clone was selected containing a plasmid designated pho3-23. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the genes are shown as SEQ ID NOS. 28 and 29.

A comparison of the amino acid sequence of pho3-23 was performed.

Homologues to the GBS pho3-23 gene product can be identified in *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Streptococcus mutans* (perM). The *S. pyogenes*, *S. pneumoniae* and *E. faecalis* homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. In *S. mutans*, the *perM* gene product has been presumptively identified as a permease, but no other information is available as to the function of this protein. Considering that the pho3-23 coding region complements the leaderless *phoA* gene, it can be concluded that the pho3-17 gene product would most probably be located extracellularly.

Example 15

A further clone was selected containing a plasmid designated pho3-24. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The

nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 30 and 31.

A comparison of the amino acid sequence of pho3-24 was performed.

Homologues to the GBS pho3-24 gene product can be identified in *Streptococcus mutans* (dltB), *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Lactobacillus casei* (dltB) and *Bacillus subtilis* (dltB). The *S. pneumoniae*, *S. pyogenes* and *E. faecalis* homologues were identified from genome sequence data and no annotations were available as to the identity of the gene or gene products. In *S. mutans*, *L. casei* and *B. subtilis*, the *dltB* gene product has been identified as being a basic membrane protein that is involved in the transport of activated D-alanine through the cell membrane. The *dltB* gene product is involved in the biosynthesis of D-alanyl-lipoteichoic acid (Heaton, M.P. and F.C. Neuhaus. 1992. J. Bacteriol. 174:4707-4717). In *L. casei* and *B. subtilis*, the *dltB* gene product is believed to contain at least 9 membrane spanning domains, indicating that the protein or portions thereof are exposed to the outside of the cell.

Example 16

A further clone was selected containing a plasmid designated pho3-29. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 32 and 33.

A comparison of the amino acid sequence of pho3-29 was performed.

Homologues to the GBS pho3-29 gene product can be identified in *Borrelia burgdorferi* (p23 or ospC), *Bacillus brevis* (owp) and *Pseudomonas aeruginosa* (oprI). Although these homologues are not related to each other, they all represent major outer surface proteins. In *B. burgdorferi*, the *ospC* gene product has been identified as being a 23-kDa protein that is the immunodominant antigen on the surface of this bacterium (Padula, S.J. *et al*. 1993. Infect. Immun.

61:5097-5105). The *owp* gene product from *B. brevis* is one of two major cell wall proteins involved in the surface layer lattice (Tsuboi, A. 1988. J. Bacteriol. 170:935-945). Finally, the *oprI* gene from *P. aeruginosa* encodes a major outer membrane lipoprotein precursor (Saint-Onge, A. *et al.* 1992. J. Gen. Microbiol. 138:733-741).

Example 17

A further clone was selected containing a plasmid designated pho3-50. This plasmid contained a gene (or part thereof), which complemented the leaderless *phoA*. The nucleotide and deduced amino acid sequences of the gene are shown as SEQ ID NOS. 34 and 35.

A comparison of the amino acid sequence of pho3-50 was performed.

Homologues to the GBS pho3-50 gene product can be identified in a variety of Streptococci (penA, pbp2B, pbpB2), *Borrelia burgdorferi (pbp2)*, *Enterococcus faecalis* (pbpC), *Staphylococcus aureus* (pbpA), *Mycobacterium leprae* (pbpB) and *Helicobacter pylori* (pbp2). In all cases, the above homologues can be identified as penicillin binding proteins (PBPs). Genes encoding penicillin binding proteins are often located in a cluster of genes associated with cell wall synthesis (Pucci, M.J. *et al.* 1997. J. Bacteriol. 179:5632-5635). Furthermore, PBPs are typically integrated into the cell wall of a bacterium with some or all of the protein being exposed on the outer bacterial surface.